EPA/OPP MICROBIOLOGY LABORATORY
ESC, Ft. Meade, MD

Standard Operating Procedure
for
Confirmatory Tuberculocidal Method for Testing Disinfectant Efficacy

SOP Number: MB-07-02

Date Revised: 09-10-02

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Date Issued: ___/___/___

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1.0 SCOPE AND APPLICATION:

1.1 This SOP describes the confirmatory AOAC method to determine the tuberculocidal efficacy of hard surface disinfectants against *Mycobacterium bovis* (BCG).

2.0 DEFINITIONS:

2.1 AOAC = AOAC INTERNATIONAL

2.2 Carrier Set = One carrier "set" is defined as the primary MPB tube containing the carrier and the two additional subculture media tubes that were seeded from the carrier's corresponding neutralizer tube. There are 10 carrier sets per product sample tested.

3.0 HEALTH AND SAFETY:

3.1 All manipulations of the test organism are required to be performed in accordance with biosafety practices stipulated in the SOP MB-01, Lab Biosafety. All *M. bovis* (BCG) manipulations are performed in a biosafety level 3 isolation laboratory (i.e., room B202 or room B207).

3.2 Disinfectants may contain a number of different active ingredients, such as heavy metals, aldehydes, peroxides, and phenol. Latex gloves and other personal protective clothing or devices must be worn during the handling of these items for purposes of activation or dilution, or efficacy testing. A chemical fume hood or other containment equipment should be used when performing tasks with concentrated products.

4.0 CAUTIONS:

4.1 To ensure the stability of the test disinfectant solution, perform testing within 3 hours of preparation.

4.2 Strict adherence to the protocol is necessary for valid test results.

4.3 Use appropriate aseptic techniques for all test procedures involving the manipulation of test organisms and associated test components.

4.4 Touching the carrier or the hook to the interior sides of the medication

tube should be avoided while the carrier is being lowered into the disinfectant and the hook is being removed.

5.0 INTERFERENCES:

5.1 Touching the interior sides of the medication tube should be avoided while the carrier is being lowered into the disinfectant and the hook is being removed. Contact with the interior sides of the medication tube may cause adhesion of test microbe cells which are not in contact with the disinfectant. This may result in re-inoculation of the carrier with the test microbe as it is being removed from the medication tube. Re-inoculation of the carrier with the test microbe can lead to false positive results.

6.0 PERSONNEL QUALIFICATIONS:

6.1 Personnel are required to be knowledgeable of the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

7.0 SPECIAL APPARATUS AND MATERIALS:

7.1 Penicylinders - porcelain, 8±1 mm OD, 6±1 mm ID, 10±1 mm length

7.2 15 mL glass tissue grinders with glass pestles (Wheaton and/or Kontes)

7.3 Spectrophotometer (Beckman DU Series 500)

7.4 Manostat Colony Counter System

7.5 21 to 25 day old cultures of *M. bovis* (BCG) grown in Modified Proskauer Beck (MPB) medium

8.0 INSTRUMENT OR METHOD CALIBRATION:

8.1 Linearity and wavelength verification of the spectrophotometer should be performed according to instructions stipulated in SOP EQ-04, Spectrophotometers.

9.0 SAMPLE HANDLING AND STORAGE:

9.1 Disinfectants are stored according to manufacturers' recommendations or at room temperature if the product label or testing parameters do not specify a storage temperature. Those disinfectants requiring activation or dilution prior to use will only be activated or diluted within three hours of testing or as specified in the product test parameters.

10.0 PROCEDURE AND ANALYSIS:

10.1 Test Culture Preparation:

10.1.1 Generation of Cultures used in Testing. Each week (during testing), select 2-4 M7H9 stock cultures with typical growth and transfer a loopful of growth into the MPB broth tubes (20 mL in 25 mm X 150 mm tubes).

10.1.2 Inoculate 12 MPB broth tubes. Growth from 1 M7H9 slope can be used to inoculate multiple MPB tubes.

10.1.3 Incubate in a slanted position without disturbing for 21-25 days at 37±1°C.

10.1.4 Using the 12, 21-25 day old cultures grown in MPB medium, inoculate approximately 20-40 25 mm X 150 mm tubes containing 20 mL of MPB. Incubate in a slanted position without disturbing for 21-25 days at 37±1°C. Record all transfers on the Organism Culture Tracking Form.

10.1.5 Depending on the amount of growth from each 21-25 day old culture, 10-20 of the cultures may be required to generate enough standardized inoculum (approx. 75 mL) for a “typical” test day. A typical test day will require 36 seeded carriers, 12 carriers per petri dish. These carriers are used for the following:

Test of 2 product samples (36 total carriers; 20 for testing, 3 for carrier counts, 13 are extras).

The additional cultures are available in the event that growth in some tubes is weak.

10.1.6 On the day of the test, using a sterile transfer loop, carefully harvest the growth from the surface of the 20 mL cultures; transferring the growth collected from an individual 20 mL culture into a sterile glass tissue grinder.

10.1.7 Add 1 mL of 0.1% Tween 80 in saline solution to each glass tissue grinder. Homogenize the culture to break up large clumps or aggregates of bacteria.

10.1.8 Add 9 mL of MPB media to the homogenized culture.

10.1.9 Using a sterile pipette, transfer the homogenized *M. bovis* (BCG) suspension from the tissue grinder to a sterile test tube. Allow the culture to settle for 10-15 minutes to allow large clumps to settle out of suspension. Using a sterile pipette, transfer and pool the culture that remains in suspension to a clean, sterile flask.

10.1.10 Measure the transmittance of the pooled culture using the Beckman DU Series 500 spectrophotometer. Dilute the pooled cultures with MPB medium until the culture gives 20.0% (±1.0%) transmittance at 650 nm. Note: Wear NIOSH certified respirators or face shields during this process.

10.1.11 If an organic soil load is specified in the test parameters for the product test, measure the culture and add the appropriate amount of soil to the flask. Swirl to mix.

10.1.12 Using a sterile 25 mL pipette, aseptically transfer 24 mL quantities of the culture into sterile 25 mm X 150 mm test tubes.

10.2 Carrier Inoculation:

10.2.1 Record the timed activities on the Time Recording Sheet

(see 16.1).

10.2.2 Product Testing: Aseptically transfer 12 clean sterile carriers using a sterile hook into each of 3 25 mm X 150 mm tubes containing 24 mL of the standardized *M. bovis* (BCG) culture (with or without organic soil).

10.2.3 Three to five cylinders can be transferred per hook. The test culture must completely cover the cylinders. If a carrier is uncovered, gently shake the tube, or move the carrier within the tube using a sterile hook.

10.2.4 Expose the carriers to the *M. bovis* (BCG) culture for 15 minutes at room temperature. After this time, remove the carriers from the culture with a sterile hook and place each on its end in a sterile glass petri dish containing a double layer of sterile Whatman No. 2 filter paper.

10.2.5 The carriers can be removed from the culture by transferring more than one on each hook. Before removal, tap the carriers against the side of the tube to remove any excess culture.

10.2.6 Once the carriers are deposited in the petri dish, they cannot tip over or touch each other. Those that do cannot be used for testing.

10.2.7 A total of 3 petri dishes will be required for 36 carriers.

10.2.8 Cover the petri dishes following the transfers and place them in a 37±1°C incubator for 30 minutes. Record the time on the Time Recording Sheet: Carrier Inoculation Steps for the Confirmatory Tuberculocidal Test (see 16.1).

10.3 Disinfectant Sample Preparation:

10.3.1 Turn on the recirculating chiller and allow the temperature of the chiller unit and the test tube water bath to equilibrate. The temperature should be 20±1°C or the temperature at

which the product is to be tested as specified in the test parameters. Record the temperatures on the Confirmatory Tuberculocidal Test Information Sheet (see 16.3).

10.3.2 Follow chain of custody guidelines for disinfectant samples as stipulated in SOP COC-01, Sample Login and Tracking.

10.3.3 Ready-to use products are tested as received; no dilution is required.

10.3.4 Prepare disinfectant samples according to the specified test parameters. Record specific test parameters on the Confirmatory Tuberculocidal Test Information Sheet (see 16.3).

10.3.5 To ensure stability, prepare the disinfectant dilutions within three hours of testing or as specified in the test parameters for the product.

10.3.6 Prepare all dilutions with appropriate glassware.

10.3.7 Prior to opening the container of a liquid product, gently shake the container and thoroughly clean the area around the cap and spout with 70% ethanol. Allow the surface to dry. Remove the cap. Do not touch the inside surface of the cap. If present, carefully remove the seal attached to the top of the spout with cooled, flamed-sterilized instruments (i.e., razor blade, forceps).

10.3.8 Pour an appropriate aliquot of the sample into a sterile beaker. Do not place a pipette or any other instrument inside the product container. Place the cap on the product container and secure tightly. From the beaker, dispense ready-to-use products directly into sterile medication tubes or initiate dilutions for diluted products.

10.3.9 For diluted products, use $\geq$1.0 mL of sample disinfectant to prepare the dilution to be tested. Use v/v dilutions for liquid products and w/v dilutions for solids. Round to two decimal

places toward a stronger product. Record the preparation on the Media Reagent Preparation Sheet (see SOP MB-10, Media and Reagents Used in Efficacy Testing).

10.3.10 Once the dilutions are prepared, dispense 10 mL aliquots of the disinfectant into 24 sterile 25 mm X 100 mm medication tubes (20 for two sample product lots tested and 4 are extra).

10.3.11 After the disinfectant has been prepared and dispensed, place these tubes in the recirculating chiller water bath for at least 10 minutes prior to the start of the assay to allow them to come to the proper temperature.

10.4 Test Procedure:

10.4.1 Record timed transfer activities on the Time Recording Sheet: Carrier Transfers for the Confirmatory Tuberculocidal Test (see 16.2).

10.4.2 Start the timer. With a sterile hook, remove one carrier from its petri dish. With the other hand, remove a medication tube containing disinfectant from the rack in the recirculating chiller water bath. At the appropriate time, carefully deposit the carrier in the medication tube. Continue transferring the required number of carriers at 30 second or one minute intervals. Addition of the carriers must be made within ± 5 seconds of the exact time of the transfer.

10.4.3 As the carriers are lowered into the medication tubes, neither the carrier nor any part of the wire hook can touch the interior sides of the tube. If the interior sides are accidentally touched, the tube number is recorded in the Notes/Comments section of the Confirmatory Tuberculocidal Test Results Sheet (see 16.4). If the cylinder eventually yields a positive result, testing must be repeated, unless other uncompromised tubes are positive.

10.4.4 The medication tubes are not shaken after the cylinders are

deposited. Once a transfer is complete, the tube is placed back into the rack in the recirculating chiller water bath. Flame the hook after each carrier transfer.

10.4.5 Expose the carriers to the disinfectant according to the exposure time noted in the test parameters.

10.4.6 Following the exposure period, transfer each carrier to a test tube containing 10 mL of the neutralizer specified in the test parameters. Drain the excess disinfectant from the carrier prior to the transfer.

10.4.7 Shake the neutralizer tube with the carrier in it thoroughly and immediately transfer the carrier to a culture tube containing 20 mL of MPB medium.

10.4.8 Once all carriers have been transferred to the MPB medium, transfer 2 mL aliquots from each neutralizer tube into two additional subculture media (Middlebrook 7H9 Broth, Kirchners Medium, TB Broth) as specified in the test parameters. This portion of the assay is not timed, but the aliquots should be transferred to the subculture media as soon as possible.

10.4.9 Incubate all tubes for 60 days at 37±1°C. If no growth or scant growth occurs after 60 days, incubate an additional 30 days before recording final results.

10.4.10 See Attachment A (Testing Footnotes and Explanations) for a list of footnotes which are used to record certain observations which occurred during testing.

10.4.11 Determine the carrier counts (bacterial carrier load) on three carriers selected at random (1 per petri dish from the carriers prepared for the product test). Enumeration will be performed as stipulated in SOP MB-04, Carrier Counts. Record results on the Carrier Count Data Sheet for AOAC Confirmatory Tuberculocidal Tests Sheet.

10.5 Recording Results:

10.5.1 Results are recorded as positive (+) or negative (0) as indicated by the presence or absence of growth. Prior to entering (+) or (0), an acid fast stain is performed (see sections 10.7.2 and 10.7.3). Record results at 60 days and again at 90 days if necessary.

10.6 Confirmation Procedures For Product Testing:

10.6.1 To confirm the results of product testing, representative positive subculture tubes from the 10 carrier sets are selected for further investigation.

10.6.2 The maximum number of tubes that is confirmed per product sample tested is 10.

10.6.3. At least one positive subculture tube for each carrier set with growth is confirmed.

10.6.4 If more than one subculture tube for a carrier set is positive, only growth in one subculture tube is confirmed.

10.6.5 If the MPB in the set is positive, it is the representative subculture tube used for confirmation. If MPB is not positive, then the order of selecting the representative subculture tubes for confirmation is: M7H9, Kirchners, and TB.

10.6.6 If growth is observed in only one carrier set, then all subculture tubes showing growth for that carrier are subject to confirmation.

10.7 Identification of *M. bovis* (BCG):

10.7.1 The confirmatory tests used to verify the identity of *M. bovis* (BCG) are acid fast staining and plating on selective media.

10.7.2 A smear for acid fast staining is taken from the selected

tubes with growth on the day that results are read. Acid fast rods are typical for *M. bovis* (BCG). The acid fast staining results should be read promptly prior to assigning a (+) or (0) to the results.

10.7.3 If acid fast rods are observed then a (+) is assigned to the results. If no cells are observed for the acid fast stain then a (0) is applied to the results.

10.7.4 In addition, growth from these positive tubes is struck over the surface of a Middlebrook 7H9 (M7H9) agar plate, a selective medium, and incubated for 21-25 days at 37±1°C.

10.7.5 Following the 21-25 day incubation period, the colony morphology of the organism on M7H9 agar should be evaluated. *M. bovis* (BCG) typically appears as colorless to buff-colored, raised, rough growth on M7H9 agar (see SOP MB-02, Test Microbes: Initiation, Maintenance and Quality Control.

10.7.6 If a satisfactory smear cannot be obtained directly from the tube, the smear for acid fast staining will be taken from the 21-25 day old M7H9 agar plate that was inoculated with the growth from the tube.

10.7.7 In the event that no cells were observed with acid fast staining initially but typical growth was observed on the M7H9, then the (0) will be corrected to read (+) on the test sheet. An entry error will be noted in the comments section of the results sheet (see 16.4).

10.7.8 Record confirmation results on the Test Microbe Confirmation Sheet (see 16.6)

11.0 DATA ANALYSIS/CALCULATIONS: None

12.0 DATA MANAGEMENT/RECORDS MANAGEMENT:

12.1 Data will be recorded promptly, legibly, and in indelible ink on the

Time Recording Sheets for Carrier Inoculation and Carrier Transfers, Confirmatory Tuberculocidal Test Information Sheet, Confirmatory Tuberculocidal Results Sheet and the Test Microbe Confirmation Sheet (see 16.0). Completed forms are archived in notebooks kept in locked file cabinets in D217. Only authorized personnel have access to the locked files. Archived data is subject to OPP's official retention schedule contained in SOP ADM-03, Records and Archives.

13.0 QUALITY CONTROL:

13.1 The OPP Microbiology Laboratory conforms to 40CFR Part 160, Good Laboratory Practices. Appropriate quality control measures are integrated into each SOP.

13.2 For quality control purposes, the required information is documented on the appropriate form(s) (see 16.0).

14.0 NONCONFORMANCE AND CORRECTIVE ACTION:

14.1 Any deviation from the standard protocol and the reason for the deviation will be recorded on the appropriate record sheet (see 16.0); corrective action will be expeditious and will be reported under the Good Laboratory Practice Statement in the final report.

15.0 REFERENCES:

15.1 Official Methods of Analysis. 1990. 15th Ed., Association of Official Analytical Chemists, Arlington, VA. Method 965.12 Part II.

16.0 FORMS AND DATA SHEETS:

16.1 Time Recording Sheet: Carrier Inoculation Steps for the Confirmatory Tuberculocidal Test

16.2 Time Recording Sheet: Carrier Transfers for the Confirmatory Tuberculocidal Test

16.3 Confirmatory Tuberculocidal Test Information Sheet

16.4 Confirmatory Tuberculocidal Results Sheet

16.5 Test Microbe Confirmation Sheet

Attachment A: Testing Footnotes

Time Recording Sheet: Carrier Inoculation Steps for the Confirmatory Tuberculocidal Test

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Type of Test (CTB, Phenol)	
Product Reg. No.	
Product Name	
Sample No(s).	

Date/Initials						
Inoculum Settle Time (from clock/and a timer)		Test Culture %T	Inoculation Time*		Dry Time**	
Start Time	End Time		Start Time	End Time	Start Time	End Time
/	/		/	/	/	/
/	/		/	/	/	/
Comments:						

* Start time=when all carriers have been transferred into the culture and End time=time when last carrier has been removed from culture (from clock/and a timer)

** Start time=when carriers are placed in the incubator and End time=when carriers are removed from the incubator (from clock)

Time Recording Sheet: Carrier Transfers for the Confirmatory Tuberculocidal Test

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____	
Test Date	
Test Type (CTB, Phenol)	
Product Reg. No.	
Product Name	

Initials/date	Drop Interval	Carrier Drop Start Time (into the disinfectant)		Carrier Drop End Time (into the neutralizer media/MPB tube)		Transfer of Neutralizer (into additional subculture media)
		Clock	Timer	Clock	Timer	Start Time[1]
Comments:						

1 Transfer of neutralizer into secondary subculture taken from the clock.

Confirmatory Tuberculocidal Test Information Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		SOP	
Name		Test Date	
Sample No.		Comments/Modifications:	
Lot No.			

TEST PARAMETERS/Confirmed by:_____			
H_2O Hardness ($CaCO_3$) ppm	Specified	Titrated(Buret)/Date/Init	HACH/Date/Init
		/ /	/ /
Use Dilution	Specified	As Prepared/Date/Init	
		/ /	
Organic Soil	Specified	As Prepared/Date/Init	
		/ /	
Neutralizer	Specified		
Temperature	Specified	Chiller Display	Test tube Water Bath
		Before: After:	Before: After:
Contact Time	Specified	As Tested	
Other Parameters	Specified		

TEST MICROBE INFORMATION/Confirmed by:_____				
Org. Control No.		21-25 Day Culture		
% Transmittance			Initiated	Harvested
Avg. CFU/Carrier		Date		

REAGENT/MEDIA INFORMATION/Confirmed by:_____			
Reagent/Media	Prep. No.	Reagent/Media	Prep. No.

Confirmatory Tuberculocidal Results Sheet
OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	

CARRIER DROP/NEUTRALIZER TRANSFER INFORMATION/Confirmed by:_____	
Carrier Drop Interval	
Analyst Dropping Carriers	
Analyst(s) Transferring Neutralizer	
Comments:	

TEST RESULTS

Date Recorded/Initials | 60 Day:_____/90Day:_____

_____

Media	1	2	3	4	5	6	7	8	9	
MPB	/	/	/	/	/	/	/	/	/	/
M7H9	/	/	/	/	/	/	/	/	/	/
Kirchners	/	/	/	/	/	/	/	/	/	/
TB Broth	/	/	/	/	/	/	/	/	/	/

Comments:

SUMMARY OF RESULTS

Date/Initials			
Number of carriers tested		Carrier sets with growth	
Carrier sets confirmed		Carrier sets with no growth	

Comments:

Test Microbe Confirmation Sheet

OPP Microbiology Laboratory

TEST INFORMATION/Confirmed by:_____			
EPA Reg. No.		Test Date	
Name		Test Organism	

Source: Tube/Plate ID	Date/ Initials	Stain Results*	Media Information			Results		
			Type	Prep. No.	Inc. Time/ Temp.	Date/ Initials	Colony Characteristics	API Test/Vitek Results (if applicable)

* Record Acid Fast or Gram Stain results as GPC=gram positive cocci, GNR=gram negative rods, AFR=acid fast rods, GPR=Gram positive rods.

Attachment A:

Testing Footnotes and Explanations
US EPA/OPP/Microbiology Laboratory

Footnote	
A	Indicates that the seeded carrier, hook, or forceps hit the interior sides of the medication tube containing disinfectant as the carrier was being dropped.
B	Indicates that the carrier was lost (dropped) during a transfer and was not recovered.
C	Indicates that a tube of a positive carrier set (one showing growth) was later determined to be a contaminant and not the test microbe. In "Comments" refer to the confirmation information for details.
D	Indicates that the primary or secondary subculture tube containing the carrier broke during vortexing. In the "Comments" indicate if carrier was recovered or if the remaining broth was placed in another tube.
E	Indicates that the carrier was exposed to the disinfectant late or early, outside of the +/- 5 second drop, spray, or wipe interval. In "Comments" indicate the approximate number of seconds outside (+/-) of the 5 second interval.
	Indicates that the carrier was placed in the neutralizer late or early, outside of the +/- 5 second drop interval. In "Comments" indicate the approximate number of seconds outside (+/-) of the 5 second interval.